Quantitative test kit for NAD-dependent histone deacetylase activity

CycLex SIRT3 Deacetylase Fluorometric Assay Kit Ver.2

100 Assays

Cat# CY-1153V2

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Intended Use

The CycLex Research Product **CycLex SIRT3 Deacetylase Fluorometric Assay Kit** detects SIRT3 activity in lysates. Primarily, the CycLex Research Product **CycLex SIRT3 Deacetylase Fluorometric Assay Kit** is designed for the rapid and sensitive evaluation of SIRT3 inhibitors or activators using crude SIRT3 fraction or purified SIRT3.

Applications for this kit include:
1) Screening inhibitors or activators of SIRT3.
2) Detecting the effects of pharmacological agents on SIRT3.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store #5. Developer and #6. Recombinant SIRT3 at -70°C and all other components below -20°C.
- Do not expose reagents to excessive light.

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Introduction

Sir2 is a conserved protein and was recently shown to regulate lifespan extension both in budding yeast and nematode. In 2000, it was reported that the yeast Sir2 protein is a NAD(+)-dependent histone deacetylase that plays a critical role in transcriptional silencing, genome stability and longevity. There are seven mammalian Sir2 homologs (1), all of which maintain the catalytic core domain of Sir2. NAD-dependent deacetylase activity has been demonstrated for mammalian SIRT1, SIRT2, SIRT3, SIRT5 and SIRT6 proteins.

The presence of NAD-dependent ADP-ribosylase and protein deacetylase activities of sirtuin proteins suggests that they may function as sensors of metabolic or oxidative states of cells and regulate cellular functions accordingly. Mammalian SIRT1, which resides in the nucleus, is the most closely related to yeast Sir2. SIRT1 binds and deacetylates p53 (2, 3), NF-kappa B (4), forkhead transcription factors (5, 6), and histones (7). SIRT1 also suppresses muscle differentiation in response to the redox state (8). SIRT2 is a cytoplasmic protein, which colocalizes with microtubules and deacetylates alpha-tubulin (9). SIRT2 abundance increases during mitosis, suggesting that the protein plays a role in cell cycle regulation (10). Human SIRT3 is a mitochondria protein, with its N-terminal 25 amino acid residues responsible for its mitochondrial localization (11, 12). Synthesized as an enzymatically inactive protein, human SIRT3 is activated by mitochondrial matrix processing peptidase to active 28-kD active enzyme (13). These observations suggest that the existence of a latent class III deacetylase that becomes catalytically activated upon import into the human mitochondria.

However, the conventional method for measuring SIRT3 activity is very complicated and laborious. In order to measure SIRT3 enzyme activity, it is necessary to prepare radioactive acetylated histone or p53 as a substrate. First, cells have to be labeled metabolically with radioactivity by adding radioactive acetic acid to the culture medium. Second, radioactive acetylated histone has to be purified from the cells. Following the reaction, it is necessary to extract and separate the radioactive acetyl group, which has been released from acetylated histone, using ethyl acetate to measure the activity of the enzyme based on the radioactivity.

Although a method for measuring the activity of deacetylase without the use of radioactive substances was reported in recent years, owing to the use of fluorescent-labeled acetylated lysine as a substrate, the reaction product must be separated from the intact substrate and the fluorescent intensity measured by reverse phase HPLC. As mentioned above, these measurement systems are difficult to adapt for processing many samples under a variety of conditions, because of their complicated operation. Thus a simple system for biochemical analysis as well as for inhibitor screening without the use of radioactive substances is preferred.

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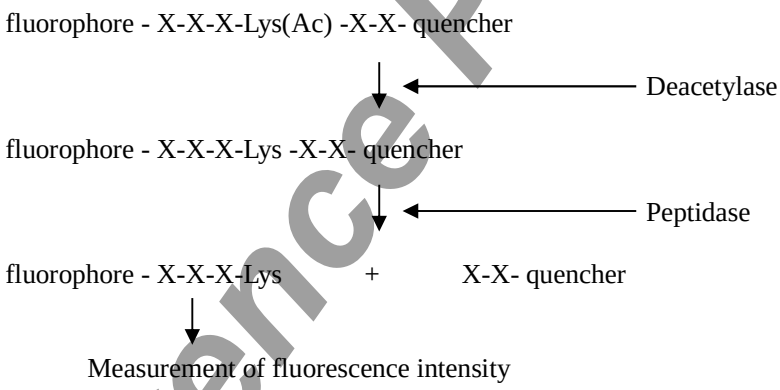
Principle of the Assay

CycLex SIRT3 Deacetylase Fluorometric Assay Kit measures the activity of SIRT3 by the basic principle of changing a SIRT3 reaction into the activity of the peptidase. In order to measure the enzyme activity of SIRT3, which is the NAD dependent Histone deacetylase, and its homolog, this kit is designed so that the activity of NAD dependent Histone deacetylase can be measured under existence of Trichostatin A, which is the powerful inhibitor of HDACs.

In this kit, fluorophore and quencher are coupled to amino terminal and carboxyl terminal of substrate peptide, respectively, and before reaction of deacetylase, the fluorescence cannot be emitted. However, if SIRT3 performs deacetylation, substrate peptide will become cut by the action of peptidase added simultaneously, quencher will separate from fluorophore, and fluorescence will be emitted. Deacetylase enzyme activity is measured by measuring this fluorescence intensity.

Since it is very simple to measure and it can be performed at a low price, the measurement of SIRT3 activity in most laboratories is possible if they are equipped with a fluorescent reader for microtiter plates. Considering that the use of fully automatic apparatus to measure fluorescence intensity has become widespread, SIRT3 activity measurement, which could not be made by the conventional method, is now possible with the CycLex SIRT3 Deacetylase Fluorometric Assay Kit using the same equipment. This new method of measurement should dramatically raise the efficiency of inhibitor screening and biochemical analysis of these enzymes.

Measuring Principle of The CycLex SIRT3 Deacetylase Fluorometric Assay Kit



***Note: This measuring principle and kit are covered under CycLex's patents.**
U.S. Patent No. 7,033,778 and No. 7256013
European Patent No. 1243658
Japanese Patent No. 4267043
Canadian Patent No. 2392711

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Materials Provided

Components of Kit

Components	Quantity	Storage
#1. SIRT3 Assay Buffer	1 mL x 2	Below -20°C
#2. Fluoro-Substrate Peptide (0.2 mM)	500 µL x 1	Below -20°C
#3. Fluoro-Deacetylated Peptide (0.2 mM)	100 µL x 1	Below -20°C
#4. NAD (2 mM)	500 µL x 1	Below -20°C
#5. Developer	500 µL x 1	-70°C
#6. Recombinant SIRT3	500 µL x 1	-70°C
#7. Stop Solution	1 mL x 2	Below -20°C
Instruction manual	1	Room temp.

Materials Required but not Provided

- **Microplate for fluorometer**
- **Microplate reading fluorometer** capable of excitation at a wavelength in the range 340-360 nm and detection of emitted light in the range 440-460 nm.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipetors with disposable tips.
- **Multi-channel pipette**
- **Microplate shaker**
- **Deionized water of the highest quality**
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Control compound(s)**

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Precautions

- Please thaw **"#2. Fluoro-Substrate Peptide"** and **"#3. Fluoro-Deacetylated Peptide"** at room temperature before use. Then, thaw the other reagents in ice and use after they are completely thawed.
- Please avoid repeated freezing and thawing of **"#5. Developer"** and **"#6. Recombinant SIRT3"**. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10-20 µL and store at -70°C.
- Please avoid mixing of protease/peptidase inhibitors such as PMSF, or alkyl amine in samples that will be measured SIRT3 activity.
- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**

NOTE: THE FOLLOWING PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER.

For research use only, not for use in diagnostic or therapeutic procedures

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Detailed Protocol

CycLex SIRT3 Deacetylase Fluorometric Assay Kit can measure the enzyme activity of SIRT3 with a homogeneous method. In this method, the reaction is initiated and the fluorescence intensity is measured by mixing simultaneously fluorescence-labeled acetylated peptide, which is a substrate, SIRT3, NAD and the developer. Since the reaction is not stopped, it is necessary to measure fluorescence intensity at regular intervals after the reaction is initiated, and to determine reaction velocity. Alternatively, within a time in which the reaction velocity is kept constant, it is also possible to stop the reaction by adding stop solution and to measure fluorescence intensity.

1. Assay Method for Measurement of SIRT3 Activity

1) Following Table.1 below, first, add **"Distilled water"**, **"#1. SIRT3 Assay Buffer"**, **"#2. Fluoro-Substrate Peptide"** and **"#4. NAD"** to microtiter plate wells. Second **"#5. Developer"** to each well of the microtiter plate and mix well.

Table.1: Reaction mixture for measurement of SIRT3 activity

Assay reagents	Enzyme Sample Assay	No Enzyme Control Assay	Positive Control Assay	No NAD Control Assay
Distilled water	**25 µL**	**25 µL**	**25 µL**	**30 µL**
#1. SIRT3 Assay Buffer	**5 µL**	**5 µL**	**5 µL**	**5 µL**
#2. Fluoro-Substrate Peptide	**5 µL**	**5 µL**	**5 µL**	**5 µL**
#4. NAD	**5 µL**	**5 µL**	**5 µL**	**-**
#5. Developer	**5 µL**	**5 µL**	**5 µL**	**5 µL**
Enzyme Sample	**5 µL**	**-**	**-**	**5 µL**
Buffer of Enzyme Sample	**-**	**5 µL**	**-**	**-**
#6. Recombinant SIRT3	**-**	**-**	**5 µL**	**-**
Total Volume of the mixture	**50 µL**	**50 µL**	**50 µL**	**50 µL**

2) Initiate reactions by adding **5 µL** of your **"Enzyme Sample"** or **"Buffer of Enzyme Sample"** or **"#6. Recombinant SIRT3"** to each well and mixing thoroughly at room temperature.

3) Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 340-360 nm and emission at 440-460 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Alternate procedure

3') While the reaction rate is kept constant, add **20 µL** of **"#7. Stop Solution"** to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader capable of excitation at a wavelength in the range 340-360 nm and detection of emitted light in the range 440-460 nm.

Note-1: During the time in which SIRT3 reaction rate is maintained, the difference in fluorescence intensity between **"Enzyme Sample Assay"** and **"No Enzyme Control Assay"** indicates the SIRT3 activity of your **"Enzyme Sample"**.

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Note-2: Although the volume of addition of **"Enzyme Sample"** or **"Buffer of Enzyme Sample"** or **"#6. Recombinant SIRT3"** is set to 5 µL in Table.1, it may be changed to a volume up to 20 µL at your discretion. In that case, please reduce the volume of **"Distilled water"** to set the final reaction volume of 50 µL.

Note-3: If enzyme samples contain some protease/peptidase able to break down **"#2. Fluoro-Substrate Peptide"**, resulting in an increase of fluorescence intensity in **"No NAD Control Assay"**, the SIRT3 activity in the samples cannot be evaluated correctly.

Note-4: If enzyme samples contain inhibitors for protease/peptidase, precise SIRT3 enzyme activity cannot be measured. Since protease/peptidase inhibitors used in the usual protein purification process strongly inhibit the peptidase activity in the development reaction, please avoid using any protease/peptidase inhibitors during the process of protein purification.

Note-5: If enzyme samples have an inhibitory effect on the peptidase in the development reaction, the final fluorescence intensity will not increase. Please use **"#3. Fluoro-Deacetylated Peptide"** instead of **"#2. Fluoro-Substrate Peptide"**, and conduct a control experiment.

2. Assay Procedures for Inhibitor/Activator Screening

1) Following Table.2 below, first, add **"Distilled water"**, **"#1. SIRT3 Assay Buffer"**, **"#2. Fluoro-Substrate Peptide"** or **"#3. Fluoro-Deacetylated Peptide"** and **"#4. NAD"** to microtiter plate wells. Second, add **"Test Compound"** or **"Solvent of Test Compound"** or **"Control Compound (not provided)"**, and **"#5. Developer"** to each well of the microtiter plate and mix well.

Table.2: Reaction mixture for inhibitor/activator screening

Assay reagents	Test Compound Assay	Solvent Control Assay	Control Compound Assay	No Enzyme Control Assay	Development Control Assay
Distilled water	**20 µL**	**20 µL**	**20 µL**	**25 µL**	**30 µL**
#1. SIRT3 Assay Buffer	**5 µL**	**5 µL**	**5 µL**	**5 µL**	**5 µL**
#2. Fluoro-Substrate Peptide	**5 µL**	**5 µL**	**5 µL**	**5 µL**	**-**
#3. Fluoro-Deacetylated Peptide	**-**	**-**	**-**	**-**	**5 µL**
#4. NAD	**5 µL**	**5 µL**	**5 µL**	**5 µL**	**-**
Test Compound	**5 µL**	**-**	**-**	**-**	**5 µL**
Solvent of Test Compound	**-**	**5 µL**	**-**	**5 µL**	**-**
Control Compound (not provided)	**-**	**-**	**5 µL**	**-**	**-**
#5. Developer	**5 µL**	**5 µL**	**5 µL**	**5 µL**	**5 µL**
#6. Recombinant SIRT3 (or Enzyme Sample)	**5 µL**	**5 µL**	**5 µL**	**-**	**-**
Total Volume of the mixture	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**

2) Initiate reactions by adding **5 µL** of **"#6. Recombinant SIRT3"** (or your **"Enzyme Sample"**) to each well and mixing thoroughly at room temperature.

3) Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 340-360 nm and emission at 440-460 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

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Alternate procedure

3') While the reaction rate is kept constant, add **20 µL** of **"#7. Stop Solution"** to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader capable of excitation at a wavelength in the range 340-360 nm and detection of emitted light in the range 440-460 nm.

Note-1: During the time in which SIRT3 reaction rate is maintained, the difference in fluorescence intensity between **"Solvent Control Assay"** and **"No Enzyme Control Assay"** indicates the SIRT3 activity.

Note-2: In order to estimate the active or inhibitory effect on SIRT3 activity by the test compounds correctly, it is necessary to conduct the control experiment of **"Solvent Control Assay"** at least once for every experiment and **"Control Compound Assay"** at least once for the first experiment, in addition to **"Test Compound Assay"** as indicated in the Table.2. When test compounds cause an active or inhibitory effect on SIRT3 activity, the level of increase of fluorescence intensity is strengthened or weakened as compared with **"Solvent Control Assay"**.

Note-3: The efficacy of the test compounds on the SIRT3 activity is the difference in fluorescence intensity between [**"Test Compound Assay"** minus **"No Enzyme Control Assay"**] and [**"Solvent Control Assay"** minus **"No Enzyme Control Assay"**].

Note-4: If test compounds have an inhibitory effect on protease/peptidase, resulting that the increase in fluorescence intensity is not or a little observed in **"Development Control Assay"**, the effect on SIRT3 activity cannot be evaluated correctly.

Note-5: Although the above tables indicate the volume of addition of **"Test Compound"** or **"Solvent of Test Compound"** or **"Control Compound (not provided)"** as 5 µL, the concentration and the volume of the reagents to add can be changed so that the concentration of test compounds becomes the setting concentration. For example, since the final volume of reaction is 50 µL here, it is also possible to add 10 µL of **"Test Compound"** or **"Solvent of Test Compound"** or **"Control Compound (not provided)"**. In this case, please reduce the volume of **"Distilled water"** to set the final reaction volume of 50 µL.

Note-6: Although the volume of addition of **"Recombinant SIRT3"** or your **"Enzyme Sample"** is set to 5 µL in above tables, it may be changed to a volume up to 20 µL at your discretion. In that case, please reduce the volume of **"Distilled water"** to set the final reaction volume of 50 µL.

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Troubleshooting

1. When chemicals that have an inhibitory effect on the peptidase are mixed in a crude SIRT3 fraction purified from various cells or the immunoprecipitate using a specific antibody against SIRT3 or other proteins, precise SIRT3 enzyme activity cannot be measured. Since the protease/peptidase inhibitors used in the usual protein purification process inhibit the peptidase activity strongly, please avoid the use of any protease/peptidase inhibitors during the protein purification process.

2. Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on SIRT3, and also when there is an inhibitory effect on the peptidase.

3. If the test reagents themselves emit fluorescence at excitation wavelength: 340-360 nm and fluorescence wavelength: 440-460 nm, the inhibitory effect of the test assay cannot be evaluated correctly.

4. The recombinant SIRT1 should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

5. The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics that are other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

6. Poor duplicates indicate inaccurate dispensing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for multi-channel pipettor maintenance.

Reagent Stability

All of the reagents included in the CycLex Research Product **CycLex SIRT3 Deacetylase Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, store the **"#5. Developer"** and **"#6. Recombinant SIRT1"** at -70°C, all other kit reagents should be stored below -20°C.

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Example of Test Results

Fig.1 Dose dependency curve of recombinant SIRT3 activity

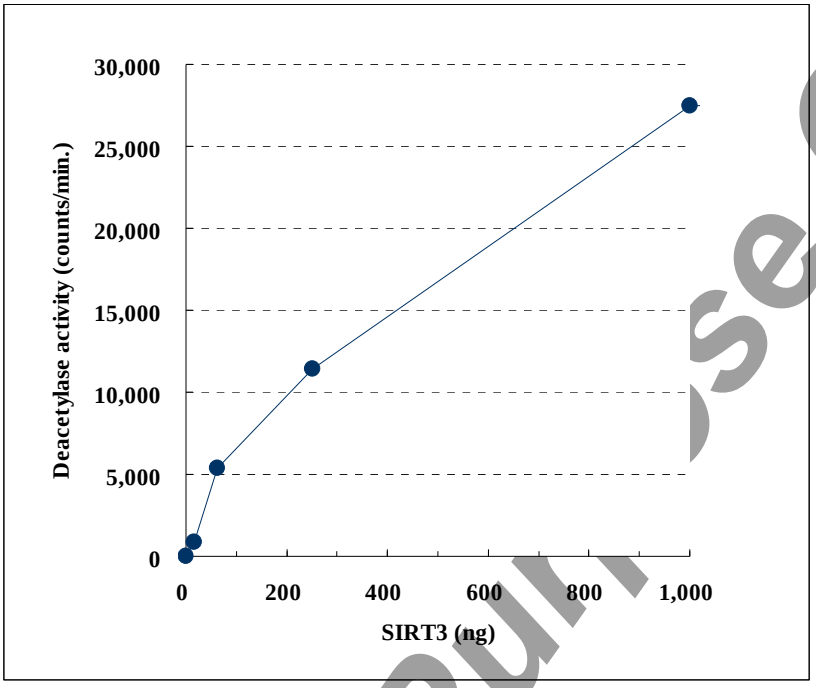

Fig.2 Time course of SIRT3-substrate deacetylation by recombinant SIRT3

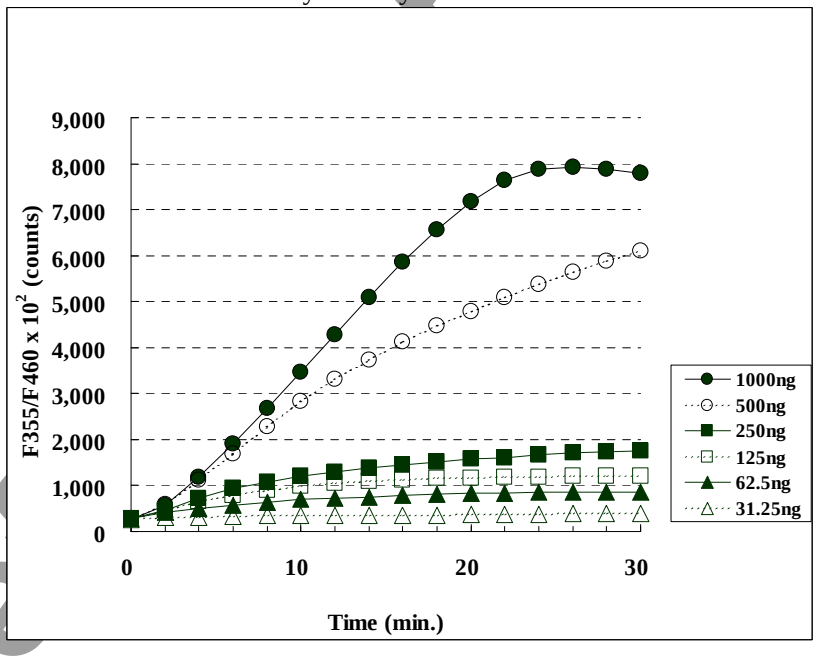

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Fig.3 Effect of Trichostatin A and NAD on recombinant SIRT3 activity

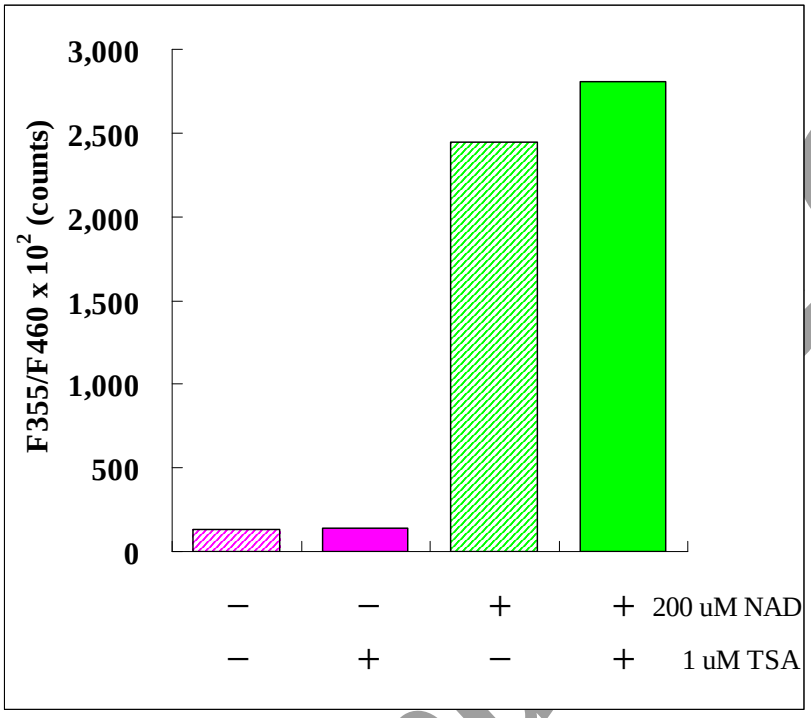

Fig.4 Km value of recombinant SIRT3 for Fluoro-Substrate Peptide

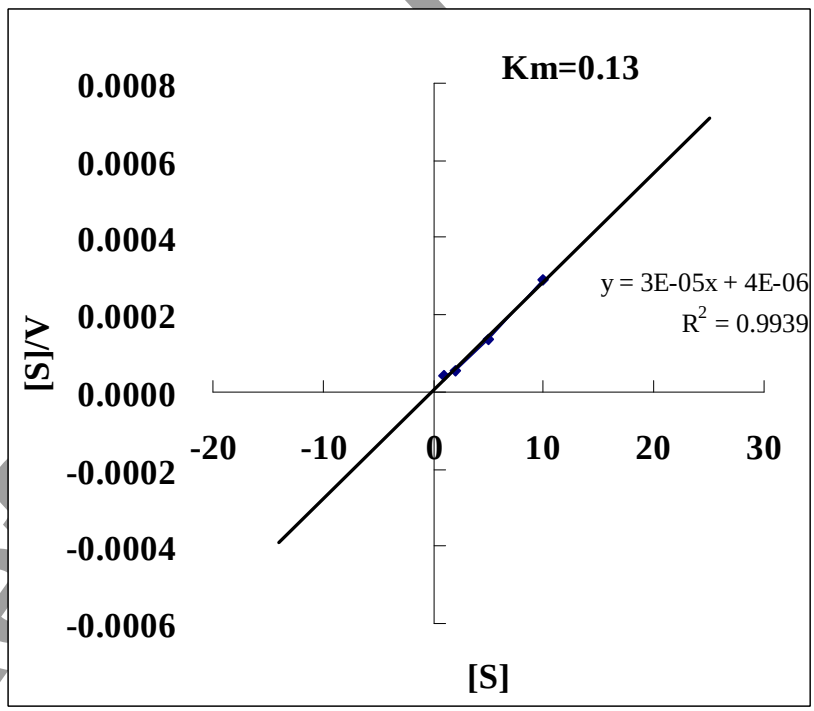

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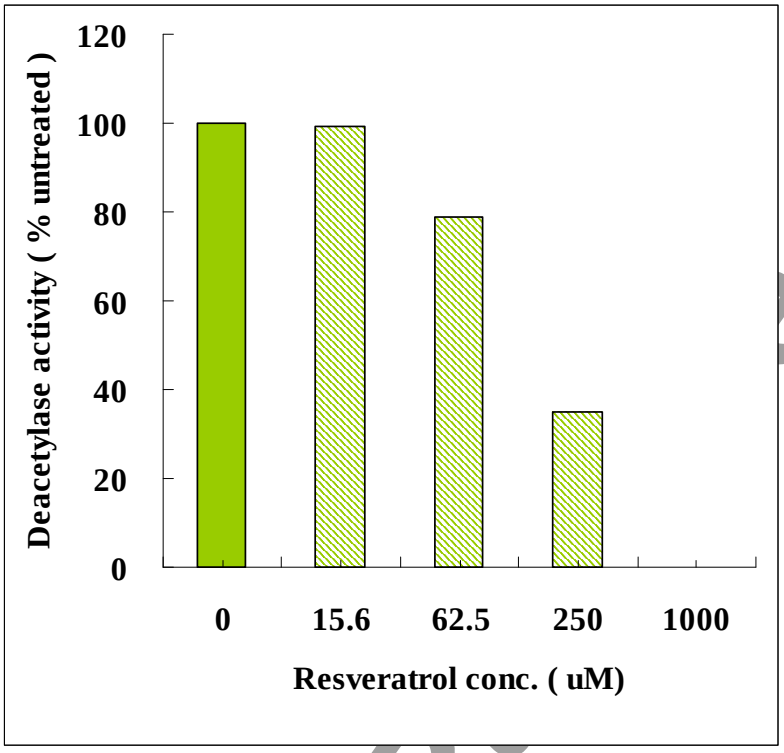
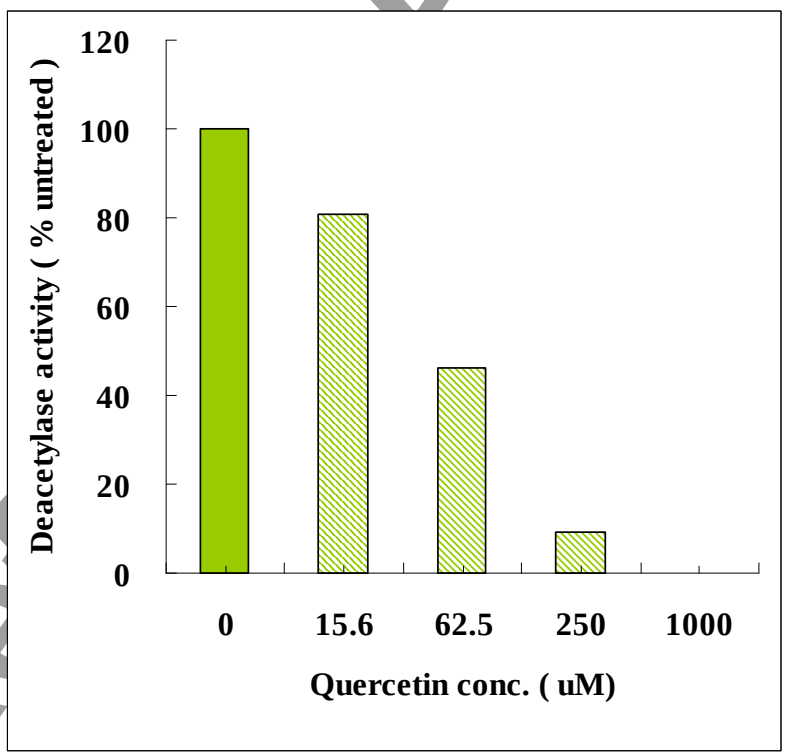
Fig.5 Effect of polyphenol on recombinant SIRT3 activity





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Related Products

*** CycLex Cellular Histone Acetylation Assay Kit: Cat# CY-1140**
*** CycLex HDACs Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1150V2**
*** CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1151V2**
*** CycLex SIRT2 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1152V2**
*** CycLex SIRT3 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1153V2**
*** CycLex SIRT6 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1156V2**
*** CycLex HDAC8 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1158V2**
*** Anti-Acetylated Histone/p53-K382 Mouse Monoclonal Antibody: Cat# CY-M1029**
*** Anti-Histone Deacetylase 1 (HDAC1) Rabbit Polyclonal Antibody: Cat# CY-P1011**
*** Anti-Histone Deacetylase 2 (HDAC2) Rabbit Polyclonal Antibody: Cat# CY-P1012**
*** Anti-Human SIRT1 Rabbit Polyclonal Antibody: Cat# CY-P1016**
*** NAD(+)-Dependent Deacetylase SIRT1: Cat# CY-E1151**
*** NAD(+)-Dependent Deacetylase SIRT2: Cat# CY-E1152**
*** NAD(+)-Dependent Deacetylase SIRT3: Cat# CY-E1153**
*** NAMPT (Nicotinamide Phosphoribosyltransferase): Cat# CY-E1251**
*** NMNAT1 (Nicotinamide Mononucleotide Adenylyltransferase 1): Cat# CY-E1252**

Note:
This product is covered under CycLex's patents.
U.S. Patent No. 7,033,778 and No. 7256013
European Patent No. 1243658
Japanese Patent No. 4267043
Canadian Patent No. 2392711

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